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Transcriptomic Analysis of Extensive Changes in Metabolic Regulation in *Kluyveromyces lactis* Strains†

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Genome-wide analysis of transcriptional regulation is generally carried out on well-characterized reference laboratory strains; hence, the characteristics of industrial isolates are therefore overlooked. In a previous study on the major cheese yeast *Kluyveromyces lactis*, we have shown that the reference strain and an industrial strain used in cheese making display a differential gene expression when grown on a single carbon source. Here, we have used more controlled conditions, i.e., growth in a fermentor with pH and oxygen maintained constant, to study how these two isolates grown in glucose reacted to an addition of lactose. The observed differences between sugar consumption and the production of various metabolites, ethanol, acetate, and glycerol, correlated with the response were monitored by the analysis of the expression of 482 genes. Extensive differences in gene expression between the strains were revealed in sugar transport, glucose repression, ethanol metabolism, and amino acid import. These differences were partly due to repression by glucose and another, yet-unknown regulation mechanism. Our results bring to light a new type of *K. lactis* strain with respect to hexose transport gene content and repression by glucose. We found that a combination of point mutations and variation in gene regulation generates a biodiversity within the *K. lactis* species that was not anticipated. In contrast to *S. cerevisiae*, in which there is a massive increase in the number of sugar transporter and fermentation genes, in *K. lactis*, interstrain diversity in adaptation to a changing environment is based on small changes at the level of key genes and cell growth control.

Kluyveromyces lactis is one of the major yeasts in cheese. It plays a role during the early steps of cheese making; by modifying the pH in the curd, it creates conditions which promote subsequent growth of bacteria. *K. lactis* is the most studied nonconventional yeast and has become an alternative model to study yeast physiology, as its physiology is very different from that of *Saccharomyces cerevisiae*. In *S. cerevisiae*, alcoholic fermentation can occur in addition to respiration in the presence of a high glucose concentration despite aerobic conditions. In contrast to *S. cerevisiae*, *K. lactis* is generally described as a strict aerobe and a Crabtree-negative yeast. Sugar transport, a well-described aspect of *K. lactis* physiology, was shown to be linked to respiration. Until now, two glucose transporters have been described, Rag1, the low-affinity transporter (7, 18, 40), and Hgt1, the high-affinity glucose transporter (2). Some *K. lactis* isolates do not possess the *RAG1* gene but, instead, a pair of genes, *KHT1* and *KHT2*, whose rearrangement corresponds to *RAG1* (38). The sensitivity to glucose repression was shown to be linked to that genetic variation, as the strains carrying the *KHT1*/*KHT2* genes are more sensitive to glucose repression than those carrying only *RAG1* (38). Many regulators of the genes encoding the hexose transporters have been identified (1–3, 7, 30).

Several studies have suggested a possible effect of the sugar transporters and/or sugar uptake on the general metabolism. Goffrini et al. (15) demonstrated the influence of *RAG1* and a glycolytic gene, *RAG2*, encoding the phosphoglucoisomerase, on respiration during growth in the presence of different sugars, leading to the definition of two groups of strains within the *K. lactis* species. Those with a Rag^+ phenotype are able to grow on glucose even when the respiration is blocked, and those with a Rag^- phenotype are not. More recently, Lemaire and Wésolowski-Louvel (24) raised the idea that the glycolysis was probably involved in the regulation of the genes encoding the glucose transporters. A slower glycolytic flux would thus provoke a reduction of the expression of the genes encoding the glucose transporters.

Although intraspecific variability at the level of the genome structure and of the DNA sequence has been shown for a number of species, it has rarely been shown that this variability affects physiological traits. Few studies have addressed the question of the variability of the global regulation of transcription of industrial yeasts. By comparing a laboratory strain and an oenological strain of *S. cerevisiae*, Brem et al. (5) and Yvert et al. (43) were able to detect and to map the variability of regulatory differences at the level of the DNA sequence between isolates. Recently, growth in copper sulfate was shown to be associated with a finite number of genes when oenological strains of *S. cerevisiae* were compared (12). With a similar approach, we found differences in gene expression, some of them in response to the carbon sources, between two isolates of *K. lactis*, CBS2359 which is used as a reference for genetics studies, and B1, an industrial strain used in cheese making (35). Although *K. lactis* is the most studied and the best known of all the nonconventional yeasts, many aspects of its physiology remain to be understood. In particular, whereas lactose is the most available carbon source in its natural environment, studies using lactose as a carbon source are very rare and

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results are contradictory (18). Here, we compare gene expression of these same two strains, in response to lactose in very controlled conditions, combining the global view of gene expression with the production of metabolites and the consumption of carbon sources. We show that the gene expression results, interpreted in relation to the physiological data, can help to understand the way by which *K. lactis* strains adapt to their environment.

MATERIALS AND METHODS

Yeast strains. *Kluyveromyces lactis* strains CBS2359 (Centraalbureau voor Schimmelcultures, Delft) and B1, an industrial strain from SOREDAB (Bongrain group) isolated from a French soft cheese (Saint Marcelin) were used in this work. Both strains display a Lac^+ phenotype. For the sake of clarity, we have called CBS2359 the laboratory strain and B1 the industrial strain throughout the text; both strains are wild isolates, although CBS2359 has been used in laboratories for a number of years.

Batch cultivation in fermentor. Precultures were grown for 48 h at 28°C in a 1-liter shake-flask containing 100 ml of YPG medium (10 g/liter Bacto peptone, 10 g/liter yeast extract, and 5 g/liter glucose). A preculture of 180 ml was used to inoculate a 20-liter bioreactor (Chemap fermentor) containing 12 liters of culture medium. The cells were cultivated in a medium containing 40 g/liter glucose, 30 g/liter yeast extract, 10 g/liter peptone, 2 g/liter K_2HPO_4, 1 g/liter KH_2PO_4, 0.05 g/liter $CaCl_2 \cdot 2H_2O$, and 3 g/liter $MgCl_2 \cdot 6H_2O$. When the biomass reached the desired concentration, 2 liters of a lactose solution was added to the bioreactor to give an initial concentration of 40 g/liter lactose. The pH was maintained at 6.0 by automatic addition of NaOH (5 N). The fermentation was performed at 20°C. The O_2 concentration was maintained above 80% of the air saturation throughout the culture.

Analytical methods. During fermentation, samples were collected every 2 h to determine biomass and the concentration of sugars, organic acids, glycerol, and ethanol. Biomass was estimated by the absorbance at 650 nm. The concentration of the metabolites in the culture supernatant was determined by high-performance liquid chromatography analysis using an HPX-87H column (Bio-Rad) maintained at 48°C with 5 mM H_2SO_4 as the eluant.

β-Galactosidase assay. β-Galactosidase activities were measured as described by Kippert (21). Cells were pelleted and resuspended in 600 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 5 mM β-mercaptoethanol) containing 0.2% sarcosyl. Samples were incubated at 30°C for 30 min. After this preincubation step, 150 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) was added and the mixtures were homogenized by gently inverting the tubes several times. When the color turned yellow, the reactions were stopped by adding 400 μl of 1.5 M Na_2CO_3. The tubes were then centrifuged for 30 s at 16,000 × *g*. The activities measured were expressed in arbitrary units.

Northern blotting. A total of 10 μg of RNAs was size fractionated by electrophoresis on a 1.2% agarose-formaldehyde gel, transferred to a Genescreen membrane (Perkin Elmer NEN) by capillarity, and then cross-linked to the membrane using UV. Probes of 409 bp and 423 bp in length were synthesized on CBS2359 genomic DNA by PCR in the genes K*lLAC12* and K*lACT1*, respectively. The primers used were KLActL (5′-CCGCTGAAAGAGAAATCGT C-3′) and KLActR (5′-AACCACCGATCCAGACAGAG-3′) and KLLac12L (5′-TTTCACTCCAATGCAATCCA-3′) and KLLac12R (5′-GCTTTTCGACA TGATCAGCA-3′). PCR amplifications were carried out in a Perkin-Elmer 2400 thermocycler in a total volume of 50 μl with 2.5 units of Ex-*Taq* DNA polymerase (Takara, Madison, WI) in the recommended buffer and about 100 ng of genomic DNA as a template. The following conditions were used: 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at the required melting temperature, and 3 min at 72°C, with a final extension step of 3 min at 72°C. The PCR products were purified on a 1% agarose gel. Radiolabeling of each probe, hybridization and radioactive signal detection, and quantification were performed as described in reference 35. Each value was normalized to the corresponding actin signal.

RNA extraction and fluorescent cDNA preparation. RNA extraction, reverse transcription, and cDNA labeling were essentially as previously described (35). Briefly, total RNA was extracted from samples using the MIDIprep RNeasy kit (QIAGEN). Reverse transcription, from 20 μg of total RNA, and cDNA direct labeling were performed using dCTP-Cy3 and dCTP-Cy5 (CyScribe first-strand cDNA labeling kit; Amersham Bioscience). RNA was then eliminated by a chemical treatment (15 min incubation with 2 M NaOH and then addition of 2 M HEPES free acid), and labeled cDNA was purified on columns and Speedvac concentrated to 5 μl, which was subsequently used for hybridization.

TABLE 1. Distribution of the 482 *K. lactis* genes represented on the microarray within the diverse cellular functions[a]

Functional category	No. of genes represented on microarray
Metabolism of various compounds	
Amino acid metabolism	67
Nitrogen and sulfur metabolism	11
Nucleotide metabolism	8
Phosphate metabolism	12
Lipid, fatty acid, and sterol metabolism	57
Vitamin, cofactor, and prosthetic group metabolism	20
Energy	
Glucose transport/regulation/repression	41
Glycolysis/neoglucogenese	17
Pentose phosphate pathway	5
Lactose/galactose metabolism	9
Tricarboxylic acid pathway	9
Respiration	16
PDH bypass[b]	10
Metabolism of energy reserves	4
Glyoxylate cycle	2
Cell cycle and DNA processing	13
Transcription	5
Protein synthesis	3
Protein metabolism	
Protein folding and stabilization	11
Protein targeting, sorting, and translocation	25
Protein modification	17
Assembly of protein complexes	10
Proteolysis	10
Intracellular organization	2
Extracellular transport	21
Intracellular transport	28
Signal transduction	4
Stress response and cell rescue	19
Ion homeostasis	5
Ion transport	12
Classification not yet clear cut	9

[a] A complete list of the genes may be found in the supplemental material.
[b] PDH, pyruvate dehydrogenase.

***K. lactis* microarray.** The microarray we used in this study was previously described (35). It contains 482 70-mer nucleotides representing *K. lactis* genes, which cover a large number of cellular functions (Table 1). Except for the *K. lactis*-specific *LAC4* and *LAC12* genes, which encode, respectively, the β-galactosidase and the lactose permease, and a gene encoding a putative sulfate transporter (KLLA0A03311g), all arrayed genes have one or more orthologues in *S. cerevisiae*, although their exact role may have diverged. For two *S. cerevisiae* genes, there exist two orthologues in *K. lactis*: KLLA0C18700g and KLLA0C18975g are the orthologues of YKL215c and KLLA0A06886g and KLLA0A06930g are the orthologues of YKR039w (which encodes a general amino acid permease).

Microarray hybridization, scanning, and image and data analysis. Prehybridization and hybridization protocols were as described previously (35). For each slide, hybridization was performed with a mixture of the cDNAs obtained from the cells grown in glucose (just before lactose addition) and the cDNAs from each sample taken 2, 4, and 20 h after addition of lactose. Cultures were performed twice independently with cytofluorophores reversed to test the statistical reproducibility. Scanning was performed using a robot Scanarray 4000 (Packard Biosciences, Boston, MA) with a resolution of 5 μm. Two images, one for each cytofluorophore, were obtained that were analyzed with the Software Quantarray (Packard BioChip Technologies), as previously described (35). Low-quality spots were flagged at this step and were thus not taken into account for the following analysis. The data obtained from image analysis were treated with the software Genespring (Silicon Genetics, Redwood City, CA). Ratios between gene expres-

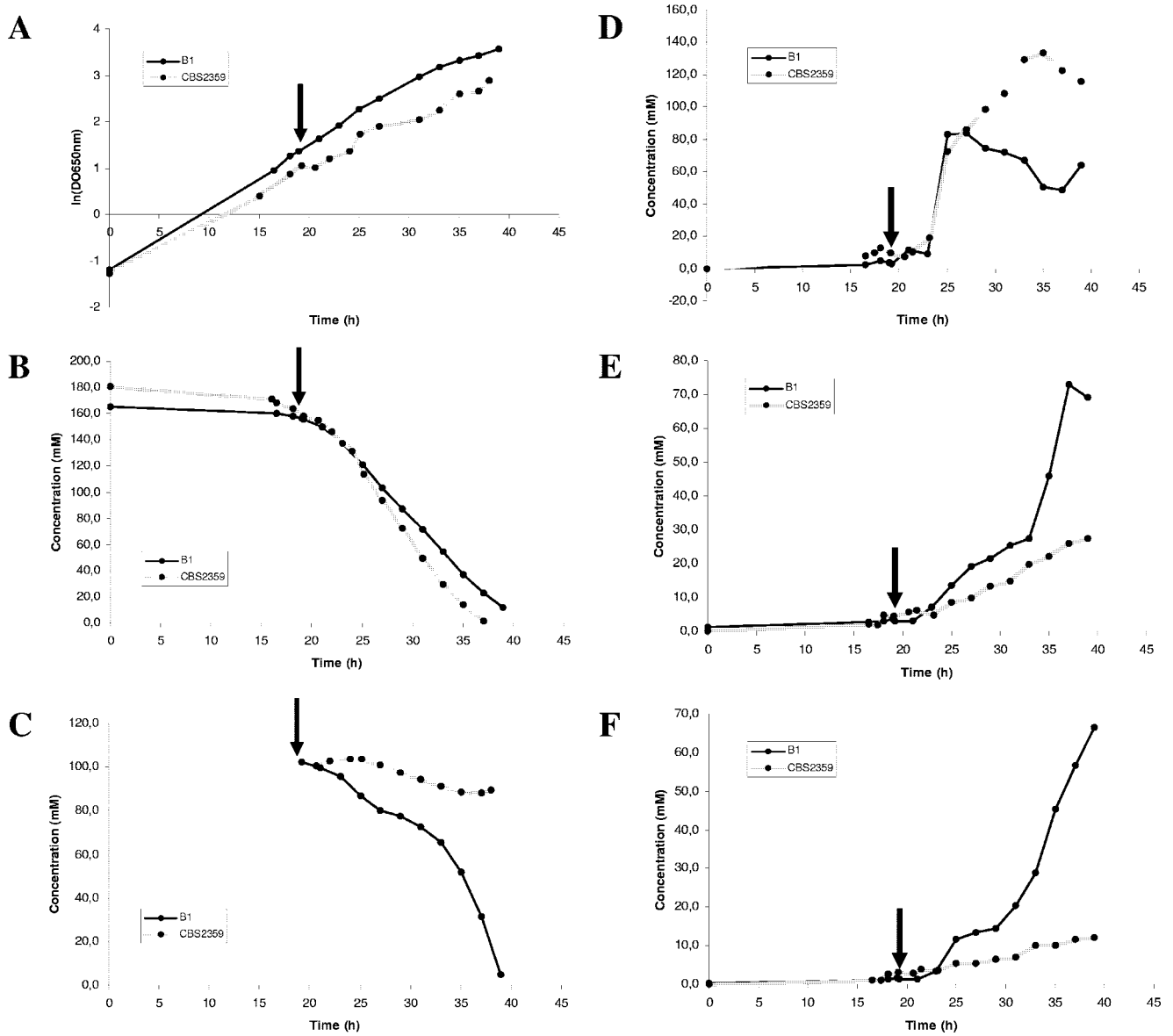

FIG. 1. Physiological data for strains CBS2359 and B1. (A) Growth of strains; (B) glucose consumption; (C) lactose consumption; (D) ethanol accumulation; (E) acetate accumulation; (F) glycerol accumulation. Arrows indicate the addition of lactose to the cultures.

sion in cultures at different times after lactose addition and gene expression in cultures before (in glucose only) were calculated. The signal/control ratios thus obtained were $\log_2$ transformed. For each culture, a control consisted of hybridizing two total cDNAs obtained from the sample in glucose only and differentially labeled (glucose Cy3/glucose Cy5). To correct the incorporation bias of both dyes, the values of the ratios thus obtained were used for normalization per gene in Genespring. The normalized values were thus statistically analyzed using the SAM software (36). For each gene and for each step, a value was assigned to represent the changes in the gene expression compared to the measure in glucose only. The probability that the expression of a given gene varies by chance was represented by a confidence value *P*. We chose the threshold *P* value of 0.05, considering that, over this value, the observed gene variation was due to chance.

RESULTS

Experimental design. Cells were inoculated into complete medium supplemented with 4% glucose. When the optical density at 650 nm (OD_{650}) reached between 10 and 12, lactose was added to a final concentration of 4%. Gene expression was compared before and after lactose addition. In parallel, physiological measurements (sugar consumption and glycerol, ethanol, and acetate production) were performed throughout the cultures. For gene expression measurements, samples were taken from each culture before lactose addition (G) and then 2 h (OD_{650} between 10 and 15), 4 h (OD_{650} between 15 and 20), and 20 h (OD_{650} between 28 and 35) after lactose addition (L_2h, L_4h, and L_20h, respectively). The G sample was used as a control to compare gene expression before and after lactose addition by calculating the ratios L_2h/G, L_4h/G, and L_20h/G.

Sugar consumption during the fermentation varies according to the strain. Figure 1 represents the growth of both strains and sugar consumption. As expected, the industrial strain

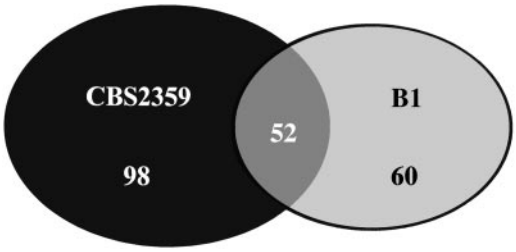

FIG. 2. Schematic representation of the number of preferentially expressed genes in each strain and in both strains 4 h after lactose addition. The number of genes whose variation in expression is significant, as described in Materials and Methods, are presented. Genes preferentially expressed in CBS2359 (black), in B1 (light gray) and in both strains (dark gray) are indicated.

seemed fitter than the laboratory strain, whose growth appeared affected by lactose (Fig. 1A). Whereas they utilize glucose at a similar rate, slightly lower in B1 (Fig. 1B), a striking difference in lactose consumption was seen, since the laboratory strain, CBS2359, did not consume lactose immediately, unlike the industrial strain, B1 (Fig. 1C). CBS2359 lactose consumption was marked by two phases: a first phase about 4 h following lactose addition, during which no lactose was consumed, and a second phase, during which little lactose was consumed, reaching 15% of the total lactose at the end of the fermentation, i.e., 20 h after lactose addition (OD_{650} between 28 and 35). Measurements carried out after glucose exhaustion showed that the lactose was finally entirely consumed in this strain after about 20 h (data not shown). During the phase of lactose consumption following glucose exhaustion, the growth rate of the strain CBS2359 was divided by six (data not shown). In strain B1, glucose and lactose consumption occurred simultaneously, but we noted variations in the rate of sugar consumption coinciding with variations in the growth rate throughout the culture. In strain CBS2359, the rate of glucose consumption reached its maximum of 0.15 mmol/g/h 4 h after lactose addition, whereas in strain B1, the rates of glucose and lactose consumption reached their maximum of 0.07 mmol/g/h and 0.3 mmol/g/h 4 and 6 h after lactose addition, respectively. Although the two strains are both Lac^+, they behaved differently in response to lactose. These results seem to indicate that strain CBS2359 is completely sensitive to repression by glucose (see Discussion).

Ethanol, acetate, and glycerol accumulation during fermentation. Cell growth was accompanied by the production of ethanol, acetate, and glycerol, as presented in (Fig. 1D, E, and F). In both strains, we observed a sudden increase in ethanol accumulation at around 4 h after lactose addition (Fig. 1D), concomitant with the moment at which the strains reach the maximal rate of sugar consumption. Interestingly, B1 accumulated 80 mM ethanol in 2 h and then began to slowly reuse it, whereas in CBS2359, the accumulation of ethanol was constant and reached 140 mM. When the glucose concentration reached 20 mM, CBS2359 started to utilize some of the accumulated ethanol (Fig. 1B and D). The amount of ethanol produced was higher in strain CBS2359 than in strain B1, while the CBS2359 biomass was lower than that of B1 (Fig. 1A and D).

Concomitant with the accumulation of ethanol, accumulation of glycerol and acetate was observed in both strains, but to different levels: the maximum amounts of glycerol and acetate were, respectively, three- and sixfold higher in B1 than in CBS2359 (Fig. 1E).

Reduced expression due to glucose repression of the lactose permease gene *LAC12* is likely responsible for the nonconsumption of lactose by CBS2359. Two hours after lactose addition, only 19 genes were up-regulated in strain B1, with a low error probability ($P < 0.03$), and 9 in the strain CBS2359, with a P value of <0.01, including the genes *LAC4*, *GAL10*, and *GAL7*, whose transcription levels were increased in both strains (25- to 40-fold, on average), reflecting an expected reaction of the cell to lactose through an induction of the lactose assimilation genes.

Four hours after lactose addition, more genes had their expression changed (Fig. 2): 112 genes in strain B1 ($P < 0.052$) and 150 genes in strain CBS2359 ($P < 0.013$) had increased levels of expression. Among them, 52 were common to both strains. The expression of the genes of the system GAL/LAC (*GAL1*, *GAL10*, *GAL7*, *GAL80*, and *LAC4*) was increased in both strains CBS2359 and B1 (Table 2). This is in agreement with the well-known induction of expression of the genes of the GAL/LAC regulon in *K. lactis* in the presence of the substrate,

TABLE 2. Relative lactose and galactose metabolism gene expression in CBS2359 and B1 4 and 20 h after lactose addition

Gene name	*S. cerevisiae* ORF[a]	KLLA_ID	L/G ratio[b] for strain at time (h) post-lactose addition:				Function
			4		20		
			CBS2359	B1	CBS2359	B1	
GAL1	YBR020w	KLLA0F08393g	9.3	21.1	≫100	1	Galactokinase
GAL10	YBR019c	KLLA0F08415g	9.7	23.1	≫100	9.6	UDP-glucose 4-epimerase and aldose 1-epimerase
GAL7	YBR018c	KLLA0F08437g	19.0	41.0	39.7	1	Galactose-1-phosphate uridylyltransferase
GAL80	YML051w	KLLA0A08162g	5.4	17.5	1	1	Galactose/lactose metabolism regulatory protein
LAC4		KLLA0B14883g	16.8	52.3	34.4	19.0	β-Galactosidase
LAC12[c]		KLLA0B14861g	1	38.9	82.0	1	Lactose permease
LAC12[c]		KLLA0B14861g	1	18.7	>100	1	Lactose permease
LAC9	YPL248c	KLLA0D12672g	1	1	1	1	Lactose regulatory protein

[a] ORF, open reading frame.
[b] Ratio of expression level of cells grown in lactose to expression level of cells grown in glucose.
[c] Corresponds to two different oligonucleotides within the *LAC12* gene.

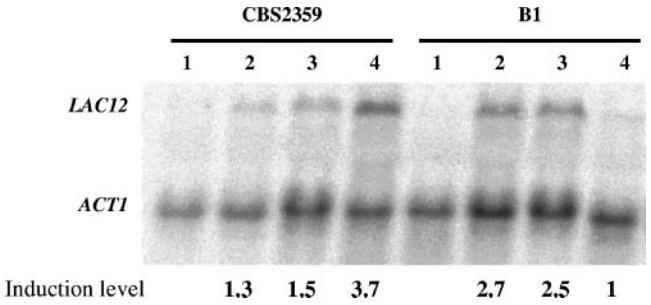

FIG. 3. Northern blot analysis of *LAC12* mRNA. Total RNAs were extracted from both strains before lactose addition (lanes 1) and 2, 4, and 20 h after lactose addition (lanes 2, 3, and 4, respectively). Around 15 μg of total RNA was separated on an agarose gel and blotted onto a nylon membrane. The probes used to detect the *HGT1* and *ACT1* genes were amplified using oligonucleotides described in Materials and Methods. The *K. lactis ACT1* gene was used as an internal reference to normalize the *HGT1* gene signal in each lane. The ratios of the normalized signals in lanes 2, 3, and 4 compared to that of lane 1 for each strain are indicated below each corresponding lane.

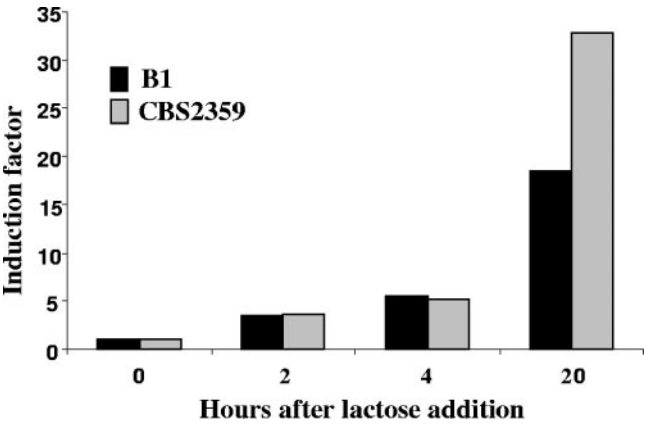

FIG. 4. Histogram of β-galactosidase activity in strains CBS2359 and B1. β-Galactosidase activity was measured as described in Materials and Methods before the addition of lactose (0) and 2 (2), 4 (4), and 20 (20) h after lactose addition. Induction factors corresponding to the ratio of activity at 0, 2, 4, or 20 h to the activity at 0 h are represented for each strain.

where the genes *GAL1* and *GAL10* have a similar induction of expression and *GAL7* is the most highly expressed gene (37).

Interestingly, 2 and 4 h after the addition of lactose, the *LAC12* gene encoding the lactose permease was increased 40-fold and over 20-fold, respectively, in strain B1, but not in CBS2359 (Table 2). This was confirmed by Northern blotting, where the *LAC12* RNA level was monitored as shown in Fig. 3. *LAC12* was nevertheless expressed in CBS 2389 at the end of the fermentation, when the glucose concentration was lower and lactose was being utilized (Fig. 3, lane 4), suggesting that glucose affects the expression of the *LAC12* gene. The lack of induction of *LAC12* gene expression by lactose is consistent with the nonconsumption of lactose by CBS2659 (Fig. 1C), but this is clearly in contradiction with the observed induction of the expression of *LAC4*, the gene encoding the β-galactosidase, which was similar to that of B1 (Table 2). We wondered if the differences in lactose consumption displayed by the two strains could also be due to a diminished β-galactosidase activity in the laboratory strain CBS2359. Figure 4 shows the results of the β-galactosidase assay during the fermentation: a threefold increase and a fivefold increase of β-galactosidase activity were observed for both strains 2 and 4 h after lactose addition, respectively. At the end of the culture, full expression of the *LAC12* gene was observed with 18- and 33-fold increases of the β-galactosidase activity in strains B1 and CBS2359, respectively. These results indicated that the nonutilization of lactose in strain CBS2359 was not due to a diminished β-galactosidase activity but rather to a difference in lactose transport through the lactose permease, consistent with the observed lack of induction of the *LAC12* gene expression in CBS2359. These results are similar to that obtained for JA6, a laboratory strain known to be sensitive to glucose repression. As CBS2359 is considered moderately sensitive to glucose repression, we sequenced the entire *LAC* regulon, including the promoter region and the *LAC9* gene, of strain B1 to search for differences in DNA sequence between the two strains. Surprisingly, except for an additional codon encoding an asparagine, as found in the *LAC9* coding sequence of the JA6 strain (22), the URS region of B1 and CBS2359 were identical. There is only a single base change at position −224 with respect to the *LAC9* start of transcription between B1 and CBS2359. This contradicts the claim that the 2-bp differences in the URS of *LAC9* between the glucose-sensitive strain JA6 and a glucose-insensitive strain were responsible for a reduced expression of *LAC9* and therefore a noninduction of *LAC12* in the former strain (23) (see discussion). We found six base changes and one insertion when the *LAC12* promoter sequences of CBS2359 and B1 were compared and a single transversion in the *LAC12* coding sequence that resulted in the replacement of a serine by an alanine at position 213. None of the base changes affected the *LAC9* binding sites, but the sequence differences observed in the promoter region may be responsible for the difference with respect to glucose repression, although these sequence changes do not affect the *LAC12* induction and activity in the absence of glucose (35) (data not shown).

Central metabolism is differently regulated in the laboratory strain and the industrial strain and involves genes that are not under glucose regulation. One of the most striking observations in our studies concerns the way the glucose metabolic pathways diverge in the two studied strains. We found three groups of genes whose expression differed between the two strains (Table 3): (i) the genes subjected to glucose repression in *S. cerevisiae* and in *K. lactis*, such as *INV1* (the *S. cerevisiae SUC2* orthologue) and *MAL22* (the *S. cerevisiae MAL32* orthologue), had a higher expression only in strain B1, (ii) the genes involved in glucose repression, such as *GSF2*, involved in glucose-dependent repression in *S. cerevisiae* (34), are highly expressed in CBS2359 only, and (iii) the genes involved in glucose utilization, such as *SWI1* and *GCY1*, which showed a 17-fold increase in B1, and *GCR1*, a positive regulator of the glycolytic genes *RAG2* (*KlPGI*), *RAG17* (*KlENO*), and *RAG6* (*KlPDC*) (29), which was induced 5-fold in strain B1 only. Overall, this confirms a differential sensitivity to glucose repression in the two strains. We also observed, in both strains, higher transcription levels for the gene *FOG1*, which encodes a protein with high similarity to the members of the *S. cerevisiae GAL83*/*SIP1*/*SIP2* gene family, whose product is involved

TABLE 3. Expression of genes involved in repression by glucose or sensitive to glucose repression in CBS2359 and B1 4 and 20 h after lactose addition

Gene name	*S. cerevisiae* ORF[a]	KLLA_ID	L/G ratio[b] for strain at time (h) post-lactose addition:				Function
			4		20		
			CBS2359	B1	CBS2359	B1	
GCR1	YPL075w	KLLA0E23507g	5.0	1	1	1	Transcriptional activator of genes involved in glycolysis
GSF2	YML048w	KLLA0A08107g	5.1	1	1	1	Involved in glucose repression
FOG1	YER027c	KLLA0B00583g	3.7	46.3	1	1	*GAL83*, Snf1 kinase complex
INV1	YIL162w	KLLA0A10417g	1	32.4	≫100	40.0	Invertase
MAL22	YBR299w	KLLA0D00231g	1	27.0	1	23.2	Maltase
SWI1	YPL016w	KLLA0D12232g	1	17.4	1	1	Component of SWI/SNF global transcription activator complex
GCY1	YOR120w	KLLA0D10109g	1	17.9	2.3	1	Galactose-induced protein of aldo/keto reductase family

[a] ORF, open reading frame.
[b] Ratio of expression level of cells grown in lactose to expression level of cells grown in glucose.

in the release from glucose repression of *LAC4* or *INV1* (17). However, the expression in B1 was 10-fold higher than in CBS22359, consistent with the other examples.

The pentose phosphate pathway generates NADPH, which is necessary for growth, and in *K. lactis*, it can replace glycolysis when the glycolytic flux is blocked. We observed an increase in the expression of only three genes involved in the pentose phosphate pathway in the laboratory strain CBS2359, *XKS1*, *RPE1*, and *GND1*, which encode the xylulokinase, the D-ribulose-5-phosphate 3-epimerase, and the 6-phosphogluconate dehydrogenase, respectively (Table 4). These results may reflect a different orientation of the metabolic flux between the strains in the presence of high concentrations of a large amount of carbon sources.

Finally, we observed that key genes involved in the glyoxylate cycle were also subject to a different regulation. Indeed, *ICL1* and *MLS1* were expressed at a very high level in the industrial strain B1 but not in the laboratory strain CBS2359 (Table 5). Lopez et al. (25) reported that *ICL1* was expressed in medium with ethanol as the sole carbon source and that it was subject to glucose repression. In *S. cerevisiae*, these two genes that are involved in the growth on ethanol are sensitive to carbon catabolite repression. In contrast to genes like *INV1* and *LAC4* whose repression is alleviated when the glucose is exhausted in CBS2359, genes like *ICL1* and *MLS1* do not show an increase in expression toward the end of the fermentation, suggesting that the regulation by glucose may be different in *K. lactis* and in *S. cerevisiae* for a number of genes. This has been noted for the *ICL1* gene with respect to the catabolite repression (25).

Microarray analysis reveals an overall difference in gene expression between strains. The gene *ADH3* encodes a mitochondrial alcohol dehydrogenase whose regulation has been the subject of several contradictory studies about its regulation. It was described as being down-regulated by ethanol (31), whereas it was claimed to be repressed by glucose and then induced by ethanol after glucose exhaustion (44). Our results, which indicate that the gene *ADH3* is not expressed during ethanol accumulation (Table 6) in CBS2359, are in agreement with those of Saliola and Falcone (31). On the other hand, *ADH3* had a higher level of expression in B1 during ethanol accumulation and in the presence of high concentrations (around 3%) of glucose; the *ADH3* pattern of expression seen in B1 (Table 6) does not, therefore, correspond to any published data for other strains. In CBS2359, the *ADH2* expression level was increased, consistent with its putative role in fermentation and its preferential expression in glucose-grown cells (32). Nevertheless, in contradiction to our results, *ADH2* was described as constitutively expressed (26). Consistent with our observation, we noted an increased expression of the gene *CCR4*, which was shown to activate the expression of *ADH2* in *S. cerevisiae* (9). We clearly demonstrated a difference in the *ADH* gene expression between the two strains. The discrepancies observed between published data and ours may be explained by the different culture conditions used; flasks were

TABLE 4. Expression of genes of the pentose phosphate pathway in CBS2359 and B1 4 and 20 h after lactose addition

Gene name	*S. cerevisiae* ORF[a]	KLLA_ID	L/G ratio[b] for strain at time (h) post-lactose addition:				Function
			4		20		
			CBS2359	B1	CBS2359	B1	
XKS1	YGR194c	KLLA0E09746g	3.5	1	1	8.4	Xylulokinase
RPE1	YJL121c	KLLA0E15136g	6.0	1	1	5.9	D-Ribulose-5-phosphate 3-epimerase
GND1	YHR183w	KLLA0A09339g	11.0	1	4.2	1	6-Phosphogluconate dehydrogenase
TAL1	YLR354c	KLLA0A02607g	1	1	2.7	1	Transaldolase
ZWF1	YNL241c	KLLA0D19855g	1	1	38.1	1	Glucose-6-phosphate 1-dehydrogenase

[a] ORF, open reading frame.
[b] Ratio of expression level of cells grown in lactose to expression level of cells grown in glucose.

TABLE 5. Expression of genes involved in anaplerotic pathways in CBS2359 and B1 4 and 20 h after lactose addition

Gene name	*S. cerevisiae* ORF[a]	KLLA_ID	L/G ratio[b] for strain at time (h) post-lactose addition:				Function
			4		20		
			CBS2359	B1	CBS2359	B1	
ICL1	YER065c	KLLA0C08107g	1	>100	1	>100	Isocitrate lyase 1
MLS1	YNL117w	KLLA0F23914g	1	52.2	1	>100	Malate synthase 1
PCK1	YKR097w	KLLA0A00484g	1	>100	48.6	>100	Phosphoenolpyruvate carboxykinase

[a] ORF, open reading frame.
[b] Ratio of expression level of cells grown in lactose to expression level of cells grown in glucose.

used in the study of Denis and Malavar (9), while we used a fermentor.

In addition to an increase in the expression of many genes involved in general biosynthetic processes, such as lipid and amino acid metabolism, we observed, in particular, a higher expression of genes involved in the metabolism of some amino acids, such as glutamate, proline, and arginine, which may reflect a high general activity in the cells at this stage of the cultures (see the supplemental material). This was accompanied by the most striking difference between the strains, with the high expression in the B1 strain of genes encoding specific and general amino acid transporters: *PUT4*, *DIP5*, and the two *K. lactis GAP1* orthologues (Table 7). On the other hand, other transporter-encoded genes like *CAN1* (amino acid permease) and *MUP1* (high-affinity methionine permease) had an increased expression in both strains, indicating that these two groups of amino acid permease-encoding genes are not under the same regulation.

It was proposed that differential gene redundancy between species reflects physiological traits (for a review, see reference 4). We examined the specific expression of two pairs of duplicated genes in *K. lactis* that are singletons in *S. cerevisiae*, YKL215c, strongly related to rat and bacterial 5 oxo-prolinase, and YKR039w, encoding the general amino acid permease *GAP1*. Considering the weaker overall gene redundancy of *K. lactis* with respect to *S. cerevisiae*, the role of these genes in the former species might be of importance. As mentioned above, there was a 30-fold difference in the expression levels of the two *GAP1* orthologues when strains were compared. The YKL215c orthologue KLLA0C18700g showed a 13-fold increase in B1 only, whereas the second YKL215c orthologue KLLA0C18879g showed a 3.5-fold increase but in CBS2359 only. It has been proposed that duplicated genes could evolve differently. While one of the paralogues keeps its primary function, the other one evolves more rapidly to perform a different role. The two *GAP1* paralogues show 91% identity at the protein sequence level, and the two YKL125c-like paralogues show 65% sequence identity. We have here the examples of (i) a pair of duplicated genes that share the same regulation but display a differential expression in the two strains studied and (ii) a pair of duplicated genes whose regulation diverged in the two strains for each copy. It will be of interest to find out, in the second example, whether the function of each duplicated gene has also diverged and if this functional divergence correlates with the observed different expression pattern.

The number of glucose transporter genes and their expression vary between strains. Glucose entry has been proposed as one of the key mechanisms of regulation of the growth and physiology of *K. lactis*, and the very limited number of glucose transporters support this hypothesis. We have seen that regulation of the lactose permease varied according to the strain. We therefore sought to determine whether the regulation of the two major *K. lactis* glucose transporters *RAG1* and *HGT1* (the low- and high-affinity glucose transporters, respectively) also showed an intraspecific variation (Table 8). Whereas CBS2359 behaves as previously observed, with an increased expression of *RAG1* by high glucose concentration and no effect of glucose on the expression for *HGT1* (2, 40), we observed, in strain B1, that *RAG1* was not induced by high glucose concentration and that *HGT1* had a large 32-fold increase in expression. Our results show that the latter gene is regulated in a different way in the two strains. We wondered if a variation

TABLE 6. Expression of genes involved in the production of metabolites in CBS2359 and B1 4 and 20 h after lactose addition

Gene name	*S. cerevisiae* ORF[a]	KLLA_ID	L/G ratio[b] for strain at time (h) post-lactose addition:				Function
			4		20		
			CBS2359	B1	CBS2359	B1	
ADH1	YOL086c	KLLA0F21010g	1	1	1	7.3	Alcohol dehydrogenase I
ADH4	YGL256w	KLLA0F13530g	1	1	1	11.9	Alcohol dehydrogenase IV, mitochondrial
ADH3	YMR083w	KLLA0B09064g	1	15.3	1	>100	Alcohol dehydrogenase III, mitochondrial
ADH2	YMR303c	KLLA0F18260g	7.8	1	3.4	1	Alcohol dehydrogenase II
ACS1	YAL054c	KLLA0A03333g	3.4	1	1	>100	Acetyl coenzyme A synthetase
DCPY	YLR044c	KLLA0E16357g	1	1	2.4	0.03	Pyruvate decarboxylase
ALD6	YPL061w	KLLA0E23144g	1	1	2.3	36.4	Aldehyde dehydrogenase

[a] ORF, open reading frame.
[b] Ratio of expression level of cells grown in lactose to expression level of cells grown in glucose.

TABLE 7. Expression of genes encoding amino acid transporters in CBS2359 and B1 4 and 20 h after lactose addition

Gene name	*S. cerevisiae* ORF[a]	KLLA_ID	L/G ratio[b] for strain at time (h) post-lactose addition:				Function
			4		20		
			CBS2359	B1	CBS2359	B1	
CAN1	YEL063c	KLLA0C02343g	4.8	3.0	44.2	1	Amino acid permease
MUP1	YGR055w	KLLA0C18568g	2.9	11.4	1	1	High-affinity methionine permease
PUT4	YOR348c	KLLA0B14685g	1	5.8	1	42.0	Proline and gamma-aminobutyrate permease
GAP1_1[c]	YKR039w	KLLA0A06886g	1	33.0	2.6	77.4	General amino acid permease
GAP1_2[c]	YKR039w	KLLA0A06930g	1	31.5	1	16.9	General amino acid permease
DIP5	YPL265w	KLLA0E16335g	1	40.3	1	1	Glutamate and aspartate permease
YKL215c_2[c]	Y215c	KLLA0C18975g	3.5	1	1	1	Oxoprolinase
YKL215c_1[c]	Y215c	KLLA0C18700g	1	13.0	1	5.5	Oxoprolinase

[a] ORF, open reading frame.
[b] Ratio of expression level of cells grown in lactose to expression level of cells grown in glucose.
[c] Corresponds to two paralogues in *K. lactis* homologous to a single *S. cerevisiae* gene.

in the DNA sequence of one or both *RAG1* and *HGT1* genes, especially in their promoter regions, or in one of their regulators, *RAG4*, could explain the different expression observed between the strains. Sequencing of the *RAG1* and *RAG4* genes, including their promoter, did not reveal any sequence variation in B1 with the sequence strain. But we discovered that a point mutation consisting of a transition from C to A at position 209 in the *HGT1* coding sequence changed a tyrosine codon into a stop codon in the industrial strain B1. This leads to a short truncated protein of 70 amino acids, rather than of 552 in the sequenced strain, that is very likely not functional in the strain B1. Our results not only show that the few hexose transporters of *K. lactis* are regulated differently according to the strain but that one of them is inactive.

DISCUSSION

In our previous work (35), using the same 482-gene microarray, we have shown differences between the *K. lactis* reference strain and an industrial strain cultivated in flasks in the presence of glucose or lactose. However several parameters may affect growth and response to the environment like limitation in oxygen (20). In the present work, we used strictly controlled oxygenation conditions to avoid such a bias. We were able to identify clear differences in the physiological response of the strains studied, and we correlated them to variations in the regulation of gene expression.

Ethanol is accumulated in the two strains but is metabolized differently. In contrast to a number of reports (see reference 20), we confirmed the observation by Gonzalez Siso et al. (19), production of ethanol in *K. lactis* approaches that of *S. cerevisiae* with a relatively high glucose concentration (1%), by noting a high ethanol production in both strains. Production of ethanol is the result of a lack of oxygen or an overflow of carbon. Considering the high oxygen supply and high glucose concentration used here, the latter explanation may be the cause of ethanol production in our experiment. In our strains, this overflow of carbon was managed differently. Both strains started accumulating ethanol suddenly 4 h after lactose addition, which may reflect an effect of lactose. As this sugar was not utilized by CBS2359, ethanol accumulation was very likely independent of the presence of lactose. Interestingly, we found that the peak of ethanol production coincided with the moment when cells reached the maximal rate of glucose consumption, consistent with our proposal. Importantly, our results have shown that the *K. lactis* strains clearly display a different regulation of ethanol metabolism; in B1, ethanol was reused a few hours after being produced, whereas it accumulated in cultures of CBS2359. This observation correlated with the differential expression of the genes encoding the alcohol dehydrogenases.

Sensitivity to glucose repression of CBS2359 partly explains the physiological differences between the two studied strains. In contrast to previous conclusions (38; see reference 39 for a review), we have shown that the reference strain CBS2359 was very sensitive to glucose repression. As a result, lactose consumption was impaired in CBS2359, whereas B1 utilized glucose and lactose concomitantly. Expression of the *GAL/LAC* genes was induced immediately after lactose addition in

TABLE 8. Expression of genes encoding glucose transporters in CBS2359 and B1 4 and 20 h after lactose addition

Gene name	*S. cerevisiae* ORF[a]	KLLA_ID	L/G ratio[b] for strain at time (h) post-lactose addition:				Function
			4		20		
			CBS2359	B1	CBS2359	B1	
HGT1	YDR342c	KLLA0A11110g	1	32.4[c]	1	1[c]	High-affinity glucose transporter
RAG1	YHR094c	KLLA0D13310g	3.6	1	50.1	1	Low-affinity glucose transporter

[a] ORF, open reading frame.
[b] Ratio of expression level of cells grown in lactose to expression level of cells grown in glucose.
[c] Pseudogene.

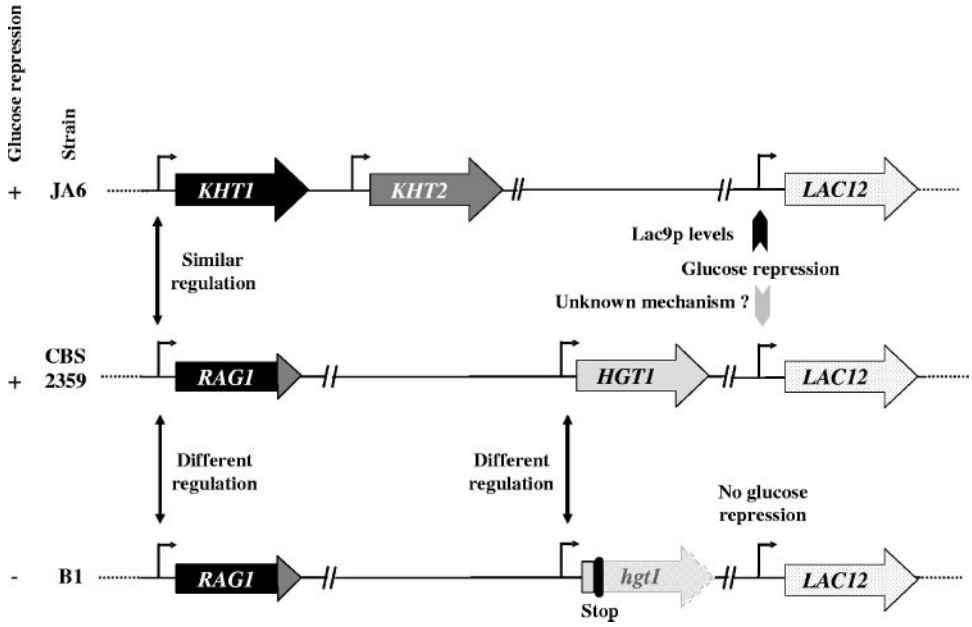

FIG. 5. Schematic representation of the genes encoding the glucose transporters Kht1p, Kht2p, Rag1p, and Hgt1p and the lactose permease Lac12p in the *K. lactis* strains JA6, CBS2359, and B1. CBS2359 and JA6 are sensitive to glucose, and their *LAC12* genes are glucose repressed, whereas in strain B1, the glucose has no effect on *LAC12*. The *RAG1* gene encoding the low-affinity glucose transporter is differently regulated in CBS2359 and B1. The *HGT1* gene encoding the high-affinity glucose transporter is inactivated in strain B1. The regulation of the different genes and the sensitivity of each strain to glucose are indicated.

CBS2359, including *LAC4*, as shown in the glucose-sensitive strain JA6 (10). Sequence comparison indicated that B1 and CBS2359 shared the same *LAC9* promoter region, whereas JA6 carried two base changes in the URS that were proposed to be responsible for the difference in response to glucose between JA6 and CBS2359 by lowering the level of Lac9p in JA6 (23). Our result clearly indicates that glucose repression does not influence *LAC9* expression in the same fashion in the two glucose-sensitive strains. Accordingly, we did not observe any difference on the level of *LAC9* expression between B1 and CBS2359, which would have been expected if *LAC9* control was similar in CBS2359 and JA6. We observed a few base changes in the B1 *LAC12* promoter that could play a role in the *LAC12* expression, but none of these changes affected the two *LAC9* binding sites. An interesting possibility is that glucose repression is effective in CBS2359 in high-oxygenation conditions and that laboratory conditions did not permit detection of this phenotypic trait, which could explain the moderate sensitivity of CBS2359 to glucose (38). Considering the intraspecific variability with respect to glucose repression, it will also be of interest to test whether what we observed for CBS2359 holds true for other "laboratory" strains.

A number of genes known to be sensitive to glucose repression were shown to be repressed in the presence of glucose in CBS2359. This was the case for several glycolytic genes and some of their regulators (Table 3). We also have observed that, independently of glucose repression regulation, genes are subject to different regulation between the two strains. This is the case of the glyoxylate cycle genes *ICL1* and *MLS1*, known to be induced when ethanol is used (8, 33). In contrast to genes like *INV1* (Table 3) and *LAC4* (Table 2), whose repression in CBS2359 is alleviated when the glucose is exhausted, they are kept repressed until the end of the fermentation. This was also seen with a number of amino acid permeases (Table 7), showing the extent of the variability of gene regulation within the *K. lactis* species. It is tempting to speculate that the clear difference between the two strains is due to an adaptation to its environment to deal with extreme conditions. Another possibility is that this set of genes entirely repressed in CBS2359 is also sensitive to lactose repression. Further work will be needed to choose between these possibilities.

Our results also confirm that regulation by glucose is different in *K. lactis* and in *S. cerevisiae*. This has been shown in a number of cases: *ICL1* gene expression with respect to catabolite repression (25), independent regulation of *INV1* by *MIG1* (13), and uncoupling of the regulation of gluconeogenesis and the glyoxylate cycle by *CAT8* (14).

A variable hexose transporter content differentiates *K. lactis* isolates. Sugar transport plays a key role in regulation of carbon metabolism and in the balance between respiration and fermentation. Our observations on the sugar transporters of the studied strains indicate that the content and regulation of these transporters vary between strains. *RAG1* was expressed at a higher level in strain CBS2359 only, in agreement with its being inducible in the presence of several hexoses, such as glucose and galactose, at concentrations above 1% (7, 40). In contrast, *HGT1* is regulated at the transcriptional level in strain B1 but not in strain CBS2359,where it had been described as constitutive in a CBS2359 derivative (2). With the discovery that B1 carries a mutation inactivating *HGT1*, our results demonstrated the existence of a new type of *K. lactis* strain with respect to glucose transport, different from the two most studied strains, CBS2359, which has *RAG1* and *HGT1*, and JA6, which has *KHT1* and *KHT2* in the place of *RAG1* but no

HGT1. This difference in glucose transporter gene complement is accompanied by differences at the level of the regulation of their expression as schematized in Fig. 5.

Several studies have shown a correlation between respiration/fermentation and sugar transport (16, 24, 27, 38), suggesting control of assimilation by fermentation due to a limitation of sugar uptake. It is notable that *HGT1* is highly inducible, as the introduction of additional sugar transporter genes was shown to allow *K. lactis* to grow in the absence of respiration (16), and it is even more notable that the *HGT1* gene is inactivated, suggesting that the mutation in *HGT1* of strain B1 may compensate for the effects of the increased *HGT1* gene expression and limit sugar uptake, thus preventing a too massive carbon uptake, which would be deleterious for the cell. This observation also suggests either that *RAG1* will have a higher basal level or that there exist other transporters in the industrial strain. In this respect, Breunig et al. (6) have mentioned that the *RAG1* locus was a rearrangement hot spot, and natural isolates with an inactivated *RAG1* gene were described previously (15).

Taken together, these observations support the idea that *K. lactis* is therefore able, by modifying the sequence and the number and the nature of the sugar transporter genes, to adapt its growth to environmental conditions. The interplay between sugar transporters seems to be very subtle. Indeed, a new hexose transporter, called Kht3p, has recently been identified whose overexpression could improve the growth of the *snf1* and *lac12* mutants on galactose but not on lactose (41).

Our results seem to reflect an adaptation of the two strains, both originating from cheese, to their environments. CBS2359 has thus adapted to glucose rather than lactose. We could suppose that glucose would have become a carbon source used preferentially by CBS2359. The mode of adaptation of *K. lactis* therefore seems to be very different from that of *S. cerevisiae*. Indeed, in the latter species, adaptation to the environment has occurred generally by duplications and accumulation of sugar transporter and fermentation genes, such as the *MEL* and *HXT* genes, in subtelomeric regions (28, 42). In *K. lactis*, the genome redundancy is reduced compared to *S. cerevisiae* (11). Since we have shown global metabolic changes and little molecular diversity, we hypothesize that, in *K. lactis*, evolutive adaptation may occur through remodeling of the transcriptional regulation combined with specific genome reshuffling rather than by chromosomal amplification. Further work on the analysis of the biodiversity within the *K. lactis* species is needed to confirm this hypothesis.

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